(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
28 December 2000 (28.12.2000)

PCT

(10) International Publication Number
WO 00/78996 A1

(51) International Patent Classification[7]: C12Q 1/18, 1/70, 1/68, C12N 15/85, 15/49

(21) International Application Number: PCT/US00/17178

(22) International Filing Date: 22 June 2000 (22.06.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
09/338,323 22 June 1999 (22.06.1999) US
09/591,899 12 June 2000 (12.06.2000) US

(71) Applicant: VIROLOGIC, INC. [US/US]; 270 East Grand Avenue, South San Francisco, CA 94080 (US).

(72) Inventors: PARKIN, Neil, T.; 1938 Bayview Avenue, Belmont, CA 94002 (US). ZIERMANN, Rainer, A.; 3908 Leona Street, San Mateo, CA 94403 (US).

(74) Agent: WHITE, John, P.; Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CU, CZ, DE, DK, DZ, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS

(57) Abstract: This invention relates to antiviral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS), particularly treatment regimens including a protease inhibitor. The invention further relates to the means and methods of monitoring the clinical progression of HIV infection and its response to antiretroviral therapy using phenotypic or genotypic susceptibility assays.

WO 00/78996 A1

MEANS AND METHODS FOR MONITORING PROTEASE INHIBITOR ANTIRETROVIRAL THERAPY AND GUIDING THERAPEUTIC DECISIONS IN THE TREATMENT OF HIV/AIDS

This application claims the benefit of U.S. Application No. 09/591,899, filed June 12, 2000 and U.S. Application No. 09/338,323, filed June 22, 1999, the contents of each of which are hereby incorporated by reference in to this application.

Throughout this application, various references are referred to within parenthesis. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

Technical Field

This invention relates to antiretroviral drug susceptibility and resistance tests to be used in identifying effective drug regimens for the treatment of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome (AIDS). The invention further relates to the means and methods of monitoring the clinical progression of HIV infection and its response to antiretroviral therapy using phenotypic or genotypic susceptibility assays. The invention also relates to novel vectors, host cells and compositions for carrying out phenotypic susceptibility tests. The invention further relates to the use of various genotypic methodologies to identify patients who do not respond to a particular antiretroviral drug regimen. This invention also relates to the screening of candidate antiretroviral

drugs for their capacity to inhibit viral replication, selected viral sequences and/or viral proteins. More particularly, this invention relates to the determination of protease inhibitor (PRI) susceptibility using phenotypic or genotypic susceptibility tests. This invention also relates to a means and method for accurately and reproducibly measuring viral replication fitness.

Background of the Invention

HIV infection is characterized by high rates of viral turnover throughout the disease process, eventually leading to CD4 depletion and disease progression. Wei X, Ghosh SK, Taylor ME, et al. (1995) Nature 343, 117-122 and Ho DD, Naumann AU, Perelson AS, et al. (1995) Nature 373, 123-126. The aim of antiretroviral therapy is to achieve substantial and prolonged suppression of viral replication. Achieving sustained viral control is likely to involve the use of sequential therapies, generally each therapy comprising combinations of three or more antiretroviral drugs. Choice of initial and subsequent therapy should, therefore, be made on a rational basis, with knowledge of resistance and cross-resistance patterns being vital to guiding those decisions. The primary rationale of combination therapy relates to synergistic or additive activity to achieve greater inhibition of viral replication. The tolerability of drug regimens will remain critical, however, as therapy will need to be maintained over many years.

In an untreated patient, some 10^{10} new viral particles are produced per day. Coupled with the failure of HIV reverse transcriptase (RT) to correct transcription errors by exonucleolytic proofreading, this high level of viral turnover results in 10^4 to 10^5 mutations per day at each position in the HIV genome. The result is the rapid establishment of extensive genotypic variation. While some template positions or base pair substitutions may be more error prone (Mansky LM, Temin HM (1995) J Virol 69, 5087-5094) (Schinazi RF, Lloyd RM, Ramanathan CS, et al. (1994) Antimicrob Agents Chemother 38, 268-274), mathematical modeling suggests that, at every possible single point, mutation may occur up to 10,000 times per day in infected individuals.

For antiretroviral drug resistance to occur, the target enzyme must be modified while preserving its function in the presence of the inhibitor. Point mutations leading to an amino acid substitution may result in changes in shape, size or charge of the active site, substrate binding site or in positions surrounding the active site of the enzyme. Mutants resistant to antiretroviral agents have been detected at low levels before the initiation of therapy. (Mohri H, Singh MK, Ching WTW, et al. (1993) Proc Natl Acad Sci USA 90, 25-29) (Nájera I, Richman DD, Olivares I, et al. (1994) AIDS Res Hum Retroviruses 10, 1479-1488) (Nájera I, Holguin A, Quiñones-Mateu E, et al. (1995) J Virol 69, 23-31). However, these mutant strains represent only a small proportion of the total viral load and may

have a replication or competitive disadvantage compared with wild-type virus. (Coffin JM (1995) Science 267, 483-489). The selective pressure of antiretroviral therapy provides these drug-resistant mutants with a competitive advantage and thus they come to represent the dominant quasi species (Frost SDW, McLean AR (1994) AIDS 8, 323-332) (Kellam P, Boucher CAB, Tijnagal JMGH (1994) J Gen Virol 75, 341-351) ultimately leading to a rebound in viral load in the patient.

Early development of antiretroviral therapy focused on inhibitors of reverse transcriptase. Both nucleoside and non-nucleoside inhibitors of this enzyme showed significant antiviral activity (DeClerq, E. (1992) AIDS Res. Hum. Retrovir. 8:119-134). However, the clinical benefit of these drugs had been limited due to drug resistance, limited potency, and host cellular factors (Richman, D.D. (1993) Ann. Rev. Pharm. Tox. 32:149-164). Thus inhibitors targeted against a second essential enzyme of HIV were urgently needed.

In 1988, the protease enzyme of HIV was crystallized and its three-dimensional structure was determined, (Navia MA, Fitzgerald PMD, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620 and Winters MA, Schapiro JM, Lawrence J, Merigan TC (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.) allowing for the rapid

development of protease inhibitors. Initially, it was hypothesized that HIV protease, unlike reverse transcriptase, would be unable to accommodate mutations leading to drug resistance. This is not the case, and to date over 20 amino acid substitutions in the HIV protease have been observed during treatment with the currently available protease inhibitors. The genetic pattern of mutations conferring resistance to these protease inhibitors is complex, and cross-resistance between structurally different compounds occurs.

PROTEASE INHIBITORS

HIV protease was classified as an aspartic proteinase on the basis of putative active-site homology (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691), its inhibition by peptastin (Richards AD, Roberts R, Dunn BM, Graves MC, Kay J (1989) FEBS Lett 247:113), and its crystal structure (Navia MA, Fitzgerald PMD, McKeever BM, Lau CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1989) Nature 337:615-620). The enzyme functions as a homodimer composed of two identical 99-amino acid chains (Debouck C, Navia MA, Fitzgerald PMD, McKeever BM, Leu CT, Heimbach JC, Herber WK, Sigal IS, Darke PL, Springer JP (1988) Proc. Natl. Acad. Sci. USA 84:8903-8906), with each chain containing the characteristic Asp-Thr-Gly active-site sequence at positions 25 to 27 (Toh H, Ono M, Saigo K, Miyata T (1985) Nature 315:691).

HIV protease processes gag (p55) and gag-pol (p160) polyprotein products into functional core proteins and viral enzymes (Kohl NE, Diehl RE, Rands E, Davis LJ, Hanobik MG, Wolanski B, Dixon RA (1991) J. Virol. 65:3007-3014 and Kramer RA, Schaber MD, Skalka AM, Ganguly K, Wong-Staal F, Reddy EP (1986) Science 231:1580-1584). During or immediately after budding, the polyproteins are cleaved by the enzyme at nine different cleavage sites to yield the structural proteins (p17, p24, p7, and p6) as well as the viral enzymes reverse transcriptase, integrase, and protease (Pettit SC, Michael SF, Swanstrom R (1993) Drug Discov. Des. 1:69-83).

An asparagine replacement for aspartic acid at active-site residue 25 results in the production of noninfectious viral particles with immature, defective cores (Huff JR (1991) AIDS J. Med. Chem. 34:2305-2314, Kaplan AH, Zack JA, Knigge M, Paul DA, Kempf DJ, Norbeck DW, Swanstrom R (1993) J. Virol. 67:4050-4055, Kohl NE, Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA, Scolnik EM, Sigal IS (1988) Proc. Natl. Acad. Sci. USA 85:4686-4690, Peng C, Ho BK, Chang TW, Chang NT (1989) J. Virol. 63:2550-2556). Similarly, wild-type virus particles produced by infected cells treated with protease inhibitors contain unprocessed precursors and are noninfectious (Crawford S, Goff SP (1985) J. Virol. 53:899-907, Gottlinger HG, Sodroski JG, Haseltine WA (1989) Proc. Natl. Acad. Sci. USA 86:5781-5785, Katoh IY, Yoshinaka Y, Rein A, Shibuya M, Odaka T, Oroszlan S (1985) Virology 145:280-292, Kohl NE,

Emini EA, Schleif WA, Davis LJ, Heimbach JC, Dixon RA, Scolnik EM, Sigal IS (1988) Proc. Natl. Acad. Sci. USA 85:4686-4690, Peng C, Ho BK, Chang TW, Chang NT (1989) J. Virol. 63:2550-2556, Stewart L, Schatz G, Wogt VM (1990) J. Virol. 64:5076-5092). Unlike reverse transcriptase inhibitors, protease inhibitors block the production of infectious virus from chronically infected cells (Lambert DM, Petteway, Jr. SR, McDanal CE, Hart TK, Leary JJ, Dreyer GB, Meek TD, Bugelski PJ, Bolognesi DP, Metcalf BW, Matthews TJ (1992) Antibicrob. Agents Chemother. 36:982-988). Although the viral protease is a symmetric dimer, it binds its natural substrates or inhibitors asymmetrically (Dreyer, GB, Boehm JC, Chenera B, DesJarlais RL, Hassell AM, Meek TD, Tomaszek TAJ, Lewis M (1993) Biochemistry 32:937-947, Miller MJ, Schneider J, Sathyanarayana BK, Toth MV, Marshall GR, Clawson L, Selk L, Kent SB, Wlodawer A (1989) Science 246:1149-1152). These findings together with the knowledge that amide bonds of proline residues are not susceptible to cleavage by mammalian endopeptidases gave rise to the first class of HIV-1 protease inhibitors based on the transition state mimetic concept, with the phenylalanine-proline cleavage site being the critical nonscissile bond (Roberts NA, Martin JA, Kinchington D, Broadhurst AV, Craig JC, Duncan IB, Galpin SA, Handa BK, Kay J, Krohn A, Lambert RW, Merett JH, Mills JS, Parkes KEB, Redshaw S, Ritchie AJ, Taylor DL, Thomas GJ, Machin PJ (1990) Science 248:358-361).

Amino acids implicated in resistance to protease inhibitors.

As new protease inhibitors are developed, the ability of certain amino acid substitutions to confer resistance to the inhibitor is usually determined by several methods, including selection of resistant strains in vitro, site-directed mutagenesis, and determination of amino acid changes that are selected during early phase clinical trials in infected patients. While some amino acid substitutions are specifically correlated with resistance to certain protease inhibitors (see below), there is considerable overlap between sets of mutations implicated in resistance to all approved protease inhibitors. Many investigators have attempted to classify these mutations as either being "primary" or "secondary", with varying definitions. For example, some investigators classify as primary mutations which are predicted, based on X-ray crystallographic data, to be in the enzyme active site with the potential for direct contact with the inhibitor (e.g. D30N, G48V, I50V, V82A/F/S/T, I84V, N88S, L90M). Secondary mutations are usually considered as being compensatory for defects in enzyme activity imposed by primary mutations, or as having enhancing effects on the magnitude of resistance imparted by the primary mutations (e.g. L10I/F/R/V, K20I/M/R/T, L24I, V32I, L33F/V, M36I/L/V, M46I/L/V, I47V, I54L/V, L63X, A71T/V, G73A/S/T, V77I, N88D). Lists of mutations and corresponding inhibitors are maintained by several organizations, for

example: Schinazi et al., Mutations in retroviral genes associated with drug resistance, *Intl. Antiviral News* 1999,7:46-69 and Shafer et al., Human Immunodeficiency Virus Reverse Transcriptase and Protease Sequence Database, Nucleic Acids Research 1999, 27(1), 348-352 (also accessible via the internet at http://www.viral-resistance.com/ or http://hivdb.stanford. edu/hiv/)

Saquinavir

Saquinavir, developed by Hoffmann-La Roche, was the first protease inhibitor to undergo clinical evaluation, demonstrating that HIV-1 protease was a valid target for the treatment of HIV infection (Jacobsen H, Brun-Vezinet F, Duncan I, Hanggi M, Ott M, Vella S, Weber J, Mous J (1994) J. Virol. 68:2016-2020). Saquinavir is a highly active peptidomimetic protease inhibitor with a 90% inhibitory concentration (IC90) of 6 nM (*id*). In vitro, saquinavir can select for variants with one or both of two amino acid substitutions in the HIV-1 protease gene, a valine-for-glycine substitution at position 48 (G48V), a methionine-for-leucine substitution at residue 90 (L90M), and the double substitution G48V-L90M (Eberle J, Bechowsky B, Rose D, Hauser U, vonder Helm K, Guertler L, Nitschko H (1995) AIDS Res. Hum. Retroviruses 11:671-676, Jacobsen H, Yasargil K, Winslow DL, Craig JC, Kroehn A, Duncan IB, Mous J (1995) Virology 206:527-534, Turriziani O, Antonelli G, Jacobsen H, Mous J, Riva E, Pistello M, Dianzani F (1994) Acta Virol. 38:297-298). In most cases, G48V is the first mutation to appear, and continued selection results in highly resistant double-mutant variants. A substitution at either residue results in a 3- to 10-fold decreased susceptibility to the inhibitor, whereas the simultaneous occurrence of both substitutions causes a more severe loss of susceptibility of >100-fold (*id*).

In vivo, saquinavir therapy appears to select almost exclusively for mutations at codons 90 and 48 (*id*,

Jacobsen H, Hangi M, Ott M, Duncan IB, Owen S, Andreoni M, Vella S, Mous J (1996) J. Infect. Dis. 173:1379-1387, Vella S, Galluzzo C, Giannini G, Pirillo MF, Duncan I, Jacobsen H, Andreoni M, Sarmati L, Ercoli L (1996) Antiviral Res. 29:91-93). Saquinavir-resistant variants emerge in approximately 45% of patients after 1 year of monotherapy with 1,800 mg daily (Craig IC, Duncan IB, Roberts NA, Whittaker L (1993) In Abstracts of the 9th International Conference on AIDS, Berlin, Germany, Duncan IB, Jacobsen H, Owen S, Roberts NA (1996) In Abstracts of the 3rd Conference of Retroviruses and Opportunistic Infections, Washington, D.D., id, Mous J, Brun-Vezinet F, Duncan IB, Haenggi M, Jacobsen H, Vella S (1994) In Abstracts of the 10th International Conference on AIDS, Yokohama, Japan). The frequency of resistance is lower (22%) in patients receiving combination therapy with zidovudine, zalcitabine, and saquinavir (Collier AC, Coombs R, Schoenfeld DA, Bassett RL, Joseph Timpone MS, Baruch A, Jones M, Facey K, Whitacre C, McAuliffe VJ, Friedman HM, Merigan TC, Reichmann RC, Hooper C, Corey L (1996) N. Engl. J. Med. 334:1011-1017). In contrast to in vitro-selected virus, where the G48V mutation is the first step to resistance, the L90M exchange is the predominant mutation selected in vivo while the G48V (2%) or the double mutant (<2%) is rarely found (id). In another recent study of in vivo resistance during saquinavir monotherapy no patient was found to harbor a G48V mutant virus (Ives KJ, Jacobsen H, Galpin SA, Garaev MM, Dorrell L, Mous J, Bragman K, Weber JN (1997 J. Antimicrob.

Chemother. 39:771-779). Interestingly, Winters et al. (id) observed a higher frequency of the G48V mutation in patients receiving higher saquinavir doses as monotherapy. All patients (six of six) who initially developed G48V also acquired a V82A mutation either during saquinavir treatment or after switching to either indinavir or nelfinavir. An identical mutational pattern was found in another study during saquinavir monotherapy (Eastman PS, Duncan IB, Gee C, Race E (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.). Some residues represent sites of natural polymorphism of the HIV-1 protease (positions 10, 36, 63, and 71) and appear to be correlated to the L90M mutation (id). Another substitution, G73S, has been recently identified and may play a role in saquinavir resistance in vivo. Isolates from five patients with early saquinavir resistance and those from two patients with induced saquinavir resistance after a switch of therapy to indinavir carried the G73S and the L90M substitutions Dulioust A, Paulous S, Guillemot L, Boue F, Galanaud P, Clavel F (1997) In Abstracts of the International Workshop on HIV Drug Resistance, Treatment Strategies and Eradication, St. Petersburg, Fla.).

Ritonavir

Ritonavir, developed by Abbott Laboratories, was the second HIV protease inhibitor to be licensed in the United States. Ritonavir is a potent and selective inhibitor of HIV protease that is derived from a C2-symmetric, peptidomimetic inhibitor (Ho DD, Toyoshima T, Mo H, Kempf DJ, Norbeck D, Chen CM, Wideburg NE, Burt SK, Erickson JW, Singh MK (1994) J. Virol. 68:2016-2020). In vitro activity has been demonstrated against a variety of laboratory strains and clinical isolates of HIV-1 with IC90s of 70 to 200 nM (Kuroda MJ, El-Farrash MA, Cloudhury S, Harada S (1995) Virology 210:212-216.

Resistant virus generated by serial in vitro passages is associated with specific mutations at positions 84, 82, 71, 63, and 46 (Markowitz M, Mo H, Kempf DJ, Norbeck DW, Bhat TN, Erickson JW, Ho DD (1995) J. Virol. 69:701-706). The I84V substitution appeared to be the major determinant of resistance, resulting in a 10-fold reduction in sensitivity to ritonavir. Addition of the V82F mutation confers an even greater level of resistance, up to 20-fold. The substitutions M46I, L63P, and A71V, when introduced into the protease coding region of wild-type NL4-3, did not result in significant changes in drug susceptibility. Based on replication kinetics experiments, these changes are likely to be compensatory for active-site mutations, restoring the impaired replicative capacity of the combined V82F and I84V mutations.

Indinavir

Indinavir, developed by Merck & Co., is the third HIV protease inhibitor licensed in the United States. Indinavir is a potent and selective inhibitor of HIV-1 and HIV-2 proteases with Ki values of 0.34 and 3.3 nM, respectively (Vacca Jp, Dorsey BD, Schleif WA, Levin RB, McDaniel SL, Darke PL, Zugay J, Quintero JC, Blahy OM, Roth E, Sardana VV, Schlabach AJ, Graham PI, Condra JH, Gotlib L, Holloway MK, Lin J, Chen L-w, Vastag K, Ostobich D, Anderson PS, Emini EA, Huff JR (1994) Proc. Natl. Acad. Sci. USA 91:4096-4100). The drug acts as peptidomimetic transition state analogue and belongs to the class of protease inhibitors known as HAPA (hydroxyaminopentane amide) compounds (ibid). Indinavir provides enhanced aqueous solubility and oral bioavailability and in cell culture exhibits an IC95 of 50 to 100 nM (Emini EA, Schleif WA, Deutsch P, Condra JH (1996) Antiviral Chemother. 4:327-331.

Despite early reports of a lack of in vitro resistance by selection with indinavir (id), Tisdale et al. (Tisdale M, Myers RE, Maschera B, Parry NR, Oliver NM, Blair ED (1995) Antibicrob. Agents Chemother. 39:1704-1710) were able to obtain resistant variants during selection in MT-4 cells with substitutions at residues 32, 46, 71, and 82. At least four mutations were required to produce a significant loss of susceptibility (6.1-fold compared with the wild type). The mutation at position 71, described as compensatory (Markowitz M, Mo H, Kempf DJ, Norbeck DW,

Bhat TN, Erickson JW, Ho DD (1995) J. Virol. (id), appeared to contribute phenotypic resistance and also to improve virus growth. Emini et al. (id) and Condra et al. (Condra JH, Holder DJ, Schleif WA, Blahy OM, Danovich RM, Gabryelski LJ, Graham DJ, Laird D, Quintero JC, Rhodes A, Robbins HL, Roth E, Shivaprakash M, Yang T, Chodakewitz JA, Deutsch PJ, Leavitt RY, Massari Fe, Mellors JW, Squires KE, Steigbigel RT, Teppler H, Emini EA (1995) Nature 374:569-571) found by constructing mutant HIV-1 clones that at least three mutations at residues 46, 63, and 82 were required for the phenotypic manifestation of resistance with a fourfold loss of susceptibility.

Nelfinavir

Nelfinavir, developed by Agouron Pharmaceuticals, is a selective, nonpeptidic HIV-1 protease inhibitor that was designed by protein structure-based techniques using iterative protein crystallographic analysis (Appelt KR, Bacquet J, Bartlett C, Booth CLJ, Freer ST, Fuhry MM, Gehring MR, Herrmann SM, Howland EF, Janson CA, Jones TR, Kan CC, Kathardekar V, Lewis KK, Marzoni GP, Mathews DA, Mohr C, Moomaw EW, Morse CA, Oatley SJ, Ogden RC, Reddy MR, Reich SH, Schoettlin WS, Smith WW, Varney MD, Villafranca JE, Ward RW, Webber S, Webber SE, Welsh KM, White J (1991) J. Med. Chem. 34:1925-1928). In vitro, nelfinavir was found to be a potent inhibitor of HIV-1 protease with a Ki of 2.0 nM (Kaldor SW, Kalish VJ, Davies JF, Shetty BV, Fritz JE, Appelt K, Burgess JA, Campanale KM, Chirgadze NY, Clawson DK, Dressman BA, Hatch SD, Khalil DA, Kosa MB, Lubbehusen PP, Muesing MA, Patrick AK, Reich SH, Su KS, Tatlock JH (1997) J. Med. Chem. 40:3979-3985). The drug demonstrated antiviral activity against several laboratory and clinical HIV-1 and HIV-2 strains with 50% effective concentrations ranging from 9 to 60 nM (Patick AK, Boritzki TJ, Bloom LA (1997) Antimicrob. Agents Chemother. 41:2159-2164). Nelfinavir exhibits additive-to-synergistic effects when combined with other antiretroviral drugs (Partaledis JA, Yamaguchi AK, Tisdale M, Blair EE, Falcione C, Maschera B, Myers RE, Pazhanisamy S, Futer O, Bullinan AB, Stuver CM, Byrn RA, Livingston DJ (1995) J. Virol. 69:5228-5235). Preclinical data showed high levels of the drug in mesenteric lymph

nodes and the spleen and good oral bioavailability (Shetty BV, Kosa MB, Khalil DA, Webber S (1996) Antimicrob. Agents Chemother. 40:110-114).

In vitro, following 22 serial passages of HIV-1_{NL4-3} in the presence of nelfinavir, a variant (P22) with a sevenfold reduced susceptibility was isolated. After an additional six passages a variant (P28) with a 30-fold-decreased susceptibility to nelfinavir was identified (Patick AK, Ho H, Markowitz M, Appelt K, Wu B, Musick L, Kaldor S, Reich S, Ho D, Webber S (1996) Antimicrob. Agents Chemother. 40:292-297). Sequence analysis of the protease gene from these variants identified in decreasing frequency the substitutions D30N, A71V, and I84V for the P22 variant and mutations M46I, I84V/A, L63P, and A71V for the P28 variant. Antiviral susceptibility testing of recombinant mutant HIV-1_{NL4-3} containing various mutations resulted in a fivefold-increased 90% effective concentration for the I84V and D30N single mutants and the M46I/I84V double mutant, whereas no change in susceptibility was observed with M46I, L63P, or A71V alone (*ibid*).

Amprenavir

Amprenavir is a novel protease inhibitor developed by Vertex Laboratories and designed from knowledge of the HIV-1 protease crystal structure (Kim EE, Baker CT, Dyer MD, Murcko MA, Rao BG, Tung RD, Navia MA (1995) J. Am. Chem. Soc. 117:1181-1182). The drug belongs to the class of sulfonamide protease inhibitors and has been shown to be a potent inhibitor of HIV-1 and HIV-2, with IC50s of 80 and 340 nM, respectively. The mean IC50 for amprenavir against clinical viral isolates was 12 nM (St. Clair MH, Millard J, Rooney J, Tisdale M, Parry N, Sadler BM, Blum MR, Painter G (1996) Antiviral Res. 29:53-56). HIV-1 variants 100-fold resistant to amprenavir have been selected by in vitro passage experiments (*id*). DNA sequence analysis of the protease of these variants revealed a sequential accumulation of point mutations resulting in amino acid substitutions L10F, M46I, I47V, and I50V. The key resistance mutation in the HIV-1 protease substrate binding site is I50V. As a single mutation it confers a two- to threefold decrease in susceptibility (ibid). The other substitutions did not result in reduced susceptibility when introduced as single mutations into an HIV-1 infectious clone (HXB2). However, a triple protease mutant clone containing the mutations M46I, I47V, and I50V was 20-fold less susceptible to amprenavir than wild-type virus. The I50V mutation has not been frequently reported in resistance studies with other HIV protease inhibitors. Kinetic characterization of these substitutions demonstrated an 80-fold reduction

in the inhibition constant (K_i) for the I50V single-mutant protease and a 270-fold-reduced K_i for the triple mutant M46I/I47V/I50V, compared to the wild-type enzyme (Pazhanisamy S, St6uvr CM, Cullinan AB, Margolin N, Rao BG (1996) J. Biol. Chem. 271:17979-17985). The single mutants L10F, M46I, and I47V did not display reduced affinity for amprenavir. The catalytic efficiency (k_{cat}/K_m) of the I50V mutant was decreased up to 25-fold, while the triple mutant M46I/I47V/I50V had a 2-fold-higher processing efficiency than the I50V single mutant, confirming the compensatory role of the M46I-and-I47V mutation. The reduced catalytic efficiency (k_{cat}/K_m) for these mutants in processing peptides appeared to be due to both increased K_m and decreased k_{cat} values.

VIRAL FITNESS

The relative ability of a given virus or virus mutant to replicate is termed viral fitness. Fitness is dependent on both viral and host factors, including the genetic composition of the virus, the host immune response, and selective pressures such as the presence of anti-viral compounds. Many drug-resistant variants of HIV-1 are less fit than the wild-type, i.e. they grow more slowly in the absence of drug selection. However, since the replication of the wild-type virus is inhibited in the presence of drug, the resistant mutant can outgrow it. The reduction in fitness may be a result of several factors including: decreased ability of the mutated enzyme (i.e. PR or RT) to recognize its natural substrates, decreased stability of

the mutant protein, or decreased kinetics of enzymatic catalysis. See Back et al., EMBO J. 15: 4040-4049, 1996; Goudsmit et al., J. Virol. 70: 5662-5664, 2996; Maschera et al., J. Biol. Chem. 271: 33231-33235, 1996; Croteau et al., J. Virol. 71: 1089-1096, 1997; Zennou et al., J. Virol. 72: 300-3306, 1998; Harrigan et al., J. Virol. 72: 3773-3778, 1998; Kosalaraksa et al., J. Virol. 73: 5356-5363, 1999; Gerondelis et al., J. Virol. 73: 5803-5813, 1999. Drug resistant viruses that are less fit than wild type may be less virulent i.e. they may cause damage to the host immune system more slowly than a wild type virus. Immunological decline may be delayed after the emergence of drug resistant mutants, compared to the rate of immunological decline in an untreated patient. The defect causing reductions in fitness may be partially or completely compensated for by the selection of viruses with additional amino acid substitutions in the same protein that bears the drug resistance mutations (for example, see Martinez-Picado et al., J. Virol. 73:3744-3752, 1999), or in other proteins which interact with the mutated enzyme. Thus, amino acids surrounding the protease cleavage site in the gag protein may be altered so that the site is better recognized by a drug-resistant protease enzyme (Doyon et al., J. Virol. 70: 3763-3769, 1996; Zhang et al., J. Virol. 71: 6662-6670, 1997; Mammano et al., J, Virol. 72: 7632-7637, 1998).

It is an object of this invention to provide a drug susceptibility and resistance test capable of showing

whether a viral population in a patient is either more or less susceptible to a given prescribed drug. Another object of this invention is to provide a test that will enable the physician to substitute one or more drugs in a therapeutic regimen for viruses that show altered susceptibility to a given drug or drugs after a course of therapy. Yet another object of this invention is to provide a test that will enable selection of an effective drug regimen for the treatment of HIV infections and/or AIDS. Yet another object of this invention is to provide the means for identifying alterations in the drug susceptibility profile of a patient's virus, in particular identifying changes in susceptibility to protease inhibitors. Still another object of this invention is to provide a test and methods for evaluating the biological effectiveness of candidate drug compounds which act on specific viruses, viral genes and/or viral proteins particularly with respect to alterations in viral drug susceptibility associated with protease inhibitors. It is also an object of this invention to provide the means and compositions for evaluating HIV antiretroviral drug resistance and susceptibility.

It is an object of this invention to provide a method for measuring replication fitness which can be adapted to viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus). This and other

objects of this invention will be apparent from the specification as a whole.

Summary of the Invention

The present invention relates to methods of monitoring, via phenotypic and genotypic methods the clinical progression of human immunodeficiency virus infection and its response to antiviral therapy. The invention is also based, in part, on the discovery that genetic changes in HIV protease (PR) which confer changes in susceptibility to antiretroviral therapy may be rapidly determined directly from patient plasma HIV RNA using phenotypic or genotypic methods. The methods utilize nucleic acid amplification based assays, such as polymerase chain reaction (PCR). Herein—after, such nucleic acid amplification based assays will be referred to as PCR based assays. This invention is based in part on the discovery of mutations at codons 10, 20, 36, 46, 63, 77 and 88 of HIV protease in PRI treated patients in which the presence of certain combinations of these mutations correlate with changes in certain PRI susceptibilities. This invention is also based on the discovery that susceptibility to HIV protease antivirals may not be altered even if primary mutations are present. Additional mutations at secondary positions in HIV protease are required for a reduction in virus susceptibility. This invention established for the first time that a mutation at position 82 of protease (V82A, F, S, or T) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was

observed in viruses with additional mutations at secondary positions, such as, 24, 71, 54, 46, 10 and/or 63 as described herein. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing V82A, F, S or T was also observed in viruses with at least 3 or more additional mutations at secondary positions. This inventions also established for the first time that a mutation at position 90 of protease (L90M) in the absence of another primary mutation was not correlated with a reduction in drug susceptibility. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was observed in viruses with additional mutations at secondary positions, such as, 73, 71, 77, and/or 10 as described herein. Decreased susceptibility to protease inhibitors, such as indinavir and saquinavir, in viruses containing L90M was also observed in viruses with at least 3 or more additional mutations at secondary positions. The mutations were found in plasma HIV nucleic acid after a period of time following the initiation of therapy. The development of these mutations, or combinations of these mutations, in HIV PR was found to be an indicator of the development of alterations in phenotypic susceptibility/resistance, which can be associated with virologic failure and subsequent immunological response.

In one embodiment of the invention, a method of assessing the effectiveness of protease antiretroviral therapy of an HIV-infected patient is provided comprising:(a)collecting

a plasma sample from the HIV-infected patient; (b) evaluating whether the plasma sample contains nucleic acid encoding HIV protease having a mutation at primary and secondary positions; and (c) determining changes in susceptibility to a protease inhibitor.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 88 from asparagine to a serine residue either alone or in combination with one or more mutations at other codons selected from the group consisting of 10, 20, 36, 46, 63 and/or 77 or a combination thereof of HIV PR. A mutation at codon 88 from an asparagine residue to a serine residue (N88S) alone correlates with an increase in susceptibility to amprenavir and a mutation at codon 88 from an asparagine residue to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof correlates with an increase in susceptibility to amprenavir and a decrease in nelfinavir and indinavir susceptibility.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at codons 10, 20, 36, 46, 63, 77, and 88 of HIV PR which correlate with changes in susceptibility to antiretroviral therapy and immunologic response. Once mutations at these loci have been detected in a patient undergoing PRI antiretroviral therapy, an alteration in the therapeutic regimen should be

considered. The timing at which a modification of the therapeutic regimen should be made, following the assessment of antiretroviral therapy using PCR based assays, may depend on several factors including the patient's viral load, CD4 count, and prior treatment history.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 82 from valine to an alanine (V82A), phenylalanine (V82F), serine (V82S), or threonine (V82T) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 20, 24, 36, 71, 54, 46, 63 and/or 10 or a combination thereof of HIV PR. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with secondary mutations at codons 24 and/or 71 or 20 and/or 36 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 82 from a valine residue to a alanine, phenylalanine, serine or threonine in combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect a substitution at codon 90 from leucine to a methionine (L90M) residue either alone or in combination with one or more mutations at other codons, referred to herein as secondary mutations, selected from the group consisting of 73, 71, 46 and/or 10 or a combination thereof of HIV PR. A mutation at codon 90 from a leucine residue to a methionine alone correlates with susceptibility to certain protease inhibitors including indinavir and saquinavir. A mutation at codon 90 from a leucine residue to a methionine in combination with secondary mutations at codons 73 and/or 71 or 73, 71 and/or 77 correlates with a reduction in susceptibility to indinavir and saquinavir, respectively. A mutation at codon 90 from a leucine residue to a methionine in combination with at least 3 secondary mutations correlates with a reduction in susceptibility to indinavir and saquinavir.

In another aspect of the invention there is provided a method for assessing the effectiveness of a protease inhibitor antiretroviral drug comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the expression of the indicator gene from step (c) with the

expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the PRI anti-HIV drug, wherein a test concentration of the PRI, anti-HIV drug is presented at steps (a) - (c); at steps (b) - (c); or at step (c).

This invention also provides a method for assessing the effectiveness of protease inhibitor antiretroviral therapy in a patient comprising: (a) developing a standard curve of drug susceptibility for an PRI anti-HIV drug; (b) determining PRI anti-HIV drug susceptibility in the patient using the susceptibility test described above; and (c) comparing the PRI anti-HIV drug susceptibility in step (b) with the standard curve determined in step (a), wherein a decrease in PRI anti-HIV susceptibility indicates development of anti-HIV drug resistance in the patient's virus and an increase in PRI anti-HIV susceptibility indicates drug hypersensitivity in the patient's virus.

This invention also provides a method for evaluating the biological effectiveness of a candidate PRI HIV antiretroviral drug compound comprising: (a) introducing a resistance test vector comprising a patient-derived segment and an indicator gene into a host cell; (b) culturing the host cell from step (a); (c) measuring expression of the indicator gene in a target host cell wherein expression of the indicator gene is dependent upon the patient derived segment; and (d) comparing the

expression of the indicator gene from step (c) with the expression of the indicator gene measured when steps (a) - (c) are carried out in the absence of the candidate PRI anti-viral drug compound, wherein a test concentration of the candidate PRI anti-viral drug compound is present at steps (a) - (c); at steps (b) - (c); or at step (c).

The expression of the indicator gene in the resistance test vector in the target cell is ultimately dependent upon the action of the HIV enzymes (PR and RT) encoded by the patient-derived segment DNA sequences. The indicator gene may be functional or non-functional.

In another aspect this invention is directed to antiretroviral drug susceptibility and resistance tests for HIV/AIDS. Particular resistance test vectors of the invention for use in the HIV/AIDS antiretroviral drug susceptibility and resistance test are identified.

Yet another aspect of this invention provides for the identification and assessment of the biological effectiveness of potential therapeutic antiretroviral compounds for the treatment of HIV and/or AIDS. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene.

Still another aspect of this invention provides for the

identification and assessment of the fitness of a virus infecting a patient. In another aspect, the invention is directed to a novel resistance test vector comprising a patient-derived segment further comprising one or more mutations on the PR gene and an indicator gene, enabling the measurement of viral fitness.

Brief Description of the Drawings

Fig. 1

Resistance Test Vector. A diagrammatic representation of the resistance test vector comprising a patient derived segment and an indicator gene.

Fig. 2

Two Cell Assay. Schematic Representation of the Assay. A resistance test vector is generated by cloning the patient-derived segment into an indicator gene viral vector. The resistance test vector is then co-transfected with an expression vector that produces amphotropic murine leukemia virus (MLV) envelope protein or other viral or cellular proteins which enable infection. Pseudotyped viral particles are produced containing the protease (PR) and the reverse transcriptase (RT) gene products encoded by the patient-derived DNA sequences. The particles are then harvested and used to infect fresh cells. Using defective PR and RT sequences it was shown that luciferase activity is dependent on functional PR and RT. PR inhibitors are added to the cells following transfection and are thus present during particle maturation. RT inhibitors, on the other hand, are added to the cells at the time of or prior to viral particle infection. The assay is performed in the absence of drug and in the presence of drug over a wide range of concentrations. Luciferase activity is determined and the percentage (%) inhibition is calculated at the different drug

concentrations tested.

Fig. 3

Examples of phenotypic drug susceptibility profiles. Data are analyzed by plotting the percent inhibition of luciferase activity vs. log10 concentration. This plot is used to calculate the drug concentration that is required to inhibit virus replication by 50% (IC50) or by 95% (IC95). Shifts in the inhibition curves towards higher drug concentrations are interpreted as evidence of drug resistance. Three typical curves for a nucleoside reverse transcriptase inhibitor (AZT), a non-nucleoside reverse transcriptase inhibitor (efavirenz), and a protease inhibitor (indinavir) are shown. A reduction in drug susceptibility (resistance) is reflected in a shift in the drug susceptibility curve toward higher drug concentrations (to the right) as compared to a baseline (pre-treatment) sample or a drug susceptible virus reference control, such as pNL4-3 or HXB-2, when a baseline sample is not available.

Fig. 4

Phenotypic PRI susceptibility profile: patient 0732. A PCR-based phenotypic susceptibility assay was carried out giving the phenotypic drug susceptibility profile showing decreased susceptibility to nelfinavir and indinavir, and increased susceptibility to amprenavir.

Fig. 5

Phenotypic PRI susceptibility profile of a protease mutant generated by site-specific oligonucleotide-directed mutagenesis. A PCR-based phenotypic susceptibility assay was carried out giving the phenotypic drug susceptibility profile of a virus having substitutions at codons 63, 77 and 88 (L63P, V77I and N88S). The profile demonstrates resistance to both nelfinavir and indinavir, and increased susceptibility to amprenavir.

Fig. A

Two Cell Fitness Assay. Schematic Representation of the Fitness Assay. A fitness test vector is generated by cloning the patient-derived segment into an indicator gene viral vector. The fitness test vector is then co-transfected with an expression vector that produces amphotropic murine leukemia virus (MLV) envelope protein or other viral or cellular proteins which enable infection. Pseudotyped viral particles are produced containing the protease (PR) and the reverse transcriptase (RT) gene products encoded by the patient-derived DNA sequences. The particles are then harvested and used to infect fresh cells. Using defective PR and RT sequences it was shown that luciferase activity is dependent on functional PR and RT. The fitness assay is typically performed in the absence of drug. If desired, the assay can also be performed at defined drug concentrations. Luciferase activity produced by patient derived viruses is compared to the luciferase activity produced by well-characterized reference viruses. Replication fitness is expressed as a percent of the reference.

Figure B.

Determining the replication fitness of patient viruses. Virus stocks produced from fitness test vectors derived from patient samples were used to infect cells. Luciferase activity was measured at various times after infection. Patient derived viruses may produce more, approximately the same, or less luciferase activity

than the reference virus (Ref) and are said to have greater, equivalent, or reduced replication fitness, respectively. The drug susceptibility profiles of three representative patient derived viruses are shown (P1, P2, P3).

Figure C.

Identifying alterations in protease or reverse transcriptase function associated with differences in replication fitness of patient viruses. Replication fitness is expressed as a percent of the reference virus (top). Fitness measurements are compared to protease processing of the p55 gag polyprotein (middle) and reverse transcriptase activity (bottom). Protease processing is measured by Western blot analysis using an antibody that reacts with the mature capsid protein (p24). The detection of unprocessed p55 or incompletely processed p41 polyproteins are indicators of reduced cleavage. Reverse transcriptase activity is measured using a quantitative RT-PCR assay and is expressed as a percent of the reference virus.

Figure D.

Correlating reduced replication fitness with reduced reverse transcriptase activity. Viruses containing various amino acid substitutions at position 190 (A, S, C, Q, E, T, V) of reverse transcriptase were constructed using site directed mutagenesis. The reference virus contains G at this position. Replication fitness and

reverse transcriptase activities were compared.

Figure E.

Correlating reduced replication fitness with reduced protease processing of p55 gag. Viruses containing various amino acid substitutions in protease (D30N, L90M, etc) were constructed using site directed mutagenesis. Replication fitness and p55 gag processing were compared.

Figure F.

Correlating reduced replication fitness with reduced drug susceptibility. A large collection (n=134)of patient samples were evaluated for phenotypic drug susceptibility and replication fitness. Replication fitness and drug susceptibility were compared.

Figure G.

Relationship between protease inhibitor susceptibility and replication fitness. Patient samples were sorted based on their replication fitness (<25% of reference, 26-75% of reference, and >75% of reference). Mean values for protease inhibitor susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

Figure H.

Relationship between reverse transcriptase inhibitor susceptibility and replication fitness. Patient samples were sorted based on their replication fitness (<25% of

reference, 26-75% of reference, and >75% of reference). Mean values for reverse transcriptase susceptibility were determined for each fitness group and plotted for each drug and all drugs combined.

Figure I.

Reduced replication fitness is associated with high numbers of protease mutations, and the L90M mutation. Patient viruses were sorted based on the number of protease mutations. Viruses with large numbers of protease mutations or the L90M protease mutation generally exhibit reduced replication fitness.

Figure J.

Low replication capacity is associated with specific protease mutations. Patient viruses were sorted based on replication capacity. Specific protease mutations either alone (D30N) or in combination (L90M plus others) were observed with high frequency in viruses with reduced replication fitness.

Figure K.

Relationship between nelfinavir susceptibility, protease processing and replication fitness. Patient viruses were sorted based on nelfinavir susceptibility (<10 or >10 of reference). Protease processing and replication fitness were plotted for all patient viruses. Viruses with reduced nelfinavir susceptibility generally exhibited reduced protease processing and reduced replication

fitness.

Figure L. Protease mutations associated with reduced protease processing. Patient viruses were sorted based on protease processing. Specific protease mutations were observed at high frequency in viruses with reduced protease processing.

Figure M.
Representative patient sample exhibiting reversion to drug susceptibility during a period of drug treatment interruption. Virus samples were collected weekly during a period of treatment interruption and evaluated for phenotypic drug susceptibility. Values shown represent fold change in susceptibility compared to the reference virus.

Figure N.
Representative patient sample exhibiting increased replication fitness during a period of drug treatment interruption. Virus samples were collected weekly during a period of treatment interruption and evaluated for phenotypic drug susceptibility. Fitness values shown represent percent of the reference virus. The increase in fitness between week 9 and week 10 corresponds to improved protease processing (bottom) and reversion of the drug resistant phenotype to a drug sensitive phenotype (Figure M).

Figure O.

Increased replication fitness during treatment interruption. Replication fitness was measured at the time of treatment interruption and various times during the period of treatment interruption. Generally, replication fitness was significantly higher in samples that corresponded to timepoints after the virus had reverted from a drug resistant phenotype to a drug sensitive phenotype.

Detailed Description of the Invention

The present invention relates to methods of monitoring the clinical progression of HIV infection in patients receiving antiretroviral therapy, particularly protease inhibitor antiretroviral therapy.

In one embodiment, the present invention provides for a method of evaluating the effectiveness of antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at one or more positions in the PR. The mutation(s) correlate positively with alterations in phenotypic susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i)

collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S). This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

In a specific embodiment, the invention provides for a

method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 46, 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon 88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 46, 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

In a specific embodiment, the invention provides for a method of evaluating the effectiveness of PRI antiretroviral therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; and (ii) determining whether the biological sample comprises nucleic acid encoding HIV PR having a mutation at codon 88 from an asparagine residue to a serine residue (N88S) either alone or in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof. This invention established, using a phenotypic susceptibility assay, that a mutation at codon

88 to a serine residue of HIV protease is correlated with an increase in amprenavir susceptibility and a mutation at codon 88 to a serine residue in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof of HIV protease are correlated with an increase in amprenavir susceptibility and a decrease in nelfinavir and indinavir susceptibility.

Under the foregoing circumstances, the phenotypic susceptibility profile and genotypic profile of the HIV virus infecting the patient has been altered reflecting a change in response to the antiretroviral agent. In the case of PRI antiretroviral therapy, the HIV virus infecting the patient may be resistant to one or more PRIs but hypersensitive to another of the PRIs as described herein. It therefore may be desirable after detecting the mutation(s), to either increase the dosage of the antiretroviral agent, change to another antiretroviral agent, or add one or more additional antiretroviral agents to the patient's therapeutic regimen. For example, if the patient was being treated with nelfinavir when the N88S mutation arose, the patient's therapeutic regimen may desirably be altered by either (i) changing to a different PRI antiretroviral agent, such as saquinavir, ritonavir or amprenavir and stopping nelfinavir treatment; or (ii) increasing the dosage of nelfinavir; or (iii) adding another antiretroviral agent to the patient's therapeutic regimen. The effectiveness of the modification in therapy may be further evaluated by monitoring viral burden such

as by HIV RNA copy number. A decrease in HIV RNA copy number correlates positively with the effectiveness of a treatment regimen.

The phrase "correlates positively," as used herein, indicates that a particular result renders a particular conclusion more likely than other conclusions.

When reference is made to a particular codon number, it is understood that the codon number refers to the position of the amino acid that the codon codes for. Therefore a codon referencing a particular number is equivalent to a "postion" referencing a particular number, such as for example, "codon 88" or "position 88".

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the

effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63 and/or 77; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63 and/or 77.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77 and/or 46 or a combination thereof; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77 and/or 46 or a combination thereof.

Another preferred, non-limiting, specific embodiment of the invention is as follows: A method of evaluating the

effectiveness of PRI therapy of a patient comprising (i) collecting a biological sample from an HIV-infected patient; (ii) purifying and converting the viral RNA to cDNA and amplifying HIV sequences using HIV primers that result in a PCR product that comprises the PR gene; (iii) performing PCR using primers that result in PCR products comprising wild type or serine at codon 88 and mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination thereof; and (iv) determining, via the products of PCR, the presence or absence of a serine residue at codon 88 and the presence or absence of mutations at codons 63, 77, 46, 10, 20, and/or 36 or a combination thereof.

The presence of the mutation at codon 88 to a serine of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention mutation at codon 88 to a serine residue increases the susceptibility to amprenavir. Using the methods of this invention, changes in the PRI therapy would be indicated.

The presence of the mutation at codon 88 to a serine of alone or in combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination thereof of HIV PR indicates that the effectiveness of the current or prospective PRI therapy may require alteration, since as shown by this invention a mutation at codon 88 to a serine residue alone increases the susceptibility to amprenavir and a mutation at codon 88 to a serine residue in

combination with mutations at condons 63, 77, 46, 10, 20, and/or 36 or a combination increases the susceptibility to amprenavir but also reduces the susceptibility to nelfinavir and indinavir. Using the methods of this invention, changes in the PRI therapy would be indicated.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes a an increase in amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63 and/or 77 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the

mutations at codon 88 to serine in combination with a mutation at codon(s) 63 and/or 77 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77 and/or 46 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 46, 63 and/or 77 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

Another preferred, non-limiting, specific embodiment of the invention is as follows: a method of evaluating the effectiveness of antiretroviral therapy of an HIV-infected patient comprising: (a) collecting a biological sample from an HIV-infected patient; and (b) determining whether the biological sample comprises nucleic acid encoding HIV

protease having a mutation at codon 88 to serine and additional mutation(s) at codons 63, 77, 46, 10, 20 and/or 36 or a combination thereof. Using the phenotypic susceptibility assay, it was observed that the presence of the mutation at codon 88 to serine of HIV PR causes an increase in amprenavir susceptibility and the presence of the mutations at codon 88 to serine in combination with a mutation at codon(s) 63, 77, 46, 10, 20 and/or 36 or a combination thereof of HIV PR causes a decrease in nelfinavir and indinavir susceptibility while increasing amprenavir susceptibility.

This invention also provides the means and methods to use the resistance test vector comprising an HIV gene and further comprising a PR mutation for drug screening. More particularly, the invention describes the resistance test vector comprising the HIV protease having a mutation at codon 88 to a serine alone or in combination with mutations at codons 10, 20, 36, 46, 63 and/or 77 or a combination thereof for drug screening. The invention further relates to novel vectors, host cells and compositions for isolation and identification of the HIV-1 protease inhibitor resistant mutant and using such vectors, host cells and compositions to carry out anti-viral drug screening. This invention also relates to the screening of candidate drugs for their capacity to inhibit said mutant.

This invention provides a method for identifying a

compound which is capable of affecting the function of the protease of HIV-1 comprising contacting the compound with the polypeptide(s) comprising all or part of the HIV-1 protease, wherein codon 88 is changed to a serine residue, wherein a positive binding indicates that the compound is capable of affecting the function of said protease.

This invention also provides a method for assessing the viral fitness of patient's virus comprising: (a) determining the luciferase activity in the absence of drug for the reference control using the susceptibility test described above; (b) determining the luciferase activity in the absence of drug for the patient virus sample using the susceptibility test described above; and (c) comparing the luciferase activity determined in step (b) with the luciferase activity determined in step (a), wherein a decrease in luciferase activity indicates a reduction in viral fitness of the patient's virus.

If a resistance test vector is constructed using a patient derived segment from a patient virus which is unfit, and the fitness defect is due to genetic alterations in the patient derived segment, then the virus produced from cells transfected with the resistance test vector will produce luciferase more slowly. This defect will be manifested as reduced luciferase activity (in the absence of drug) compared to the drug sensitive reference control, and may be expressed as a percentage of the control.

In a further embodiment of the invention, PCR based assays, including phenotypic and genotypic assays, may be used to detect mutations at positions 20 and 88 of HIV PR, which correlate with a reduction in viral fitness and immunological response.

It is a further embodiment of this invention to provide a means and method for measuring replication fitness for viruses, including, but not limited to human immunodeficiency virus (HIV), hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus).

This invention further relates to a means and method for measuring the replication fitness of HIV-1 that exhibits reduced drug susceptibility to reverse transcriptase inhibitors and protease inhibitors.

In a further embodiment of the invention , a means and methods are provided for measuring replication fitness for other classes of inhibitors of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

This invention relates to a means and method for identifying mutations in protease or reverse transcriptase that alter replication fitness.

In a further embodiment of the invention , a means and methods are provided for identifying mutations that alter replication fitness for other components of HIV-1 replication, including, but not limited to integration, virus assembly, and virus attachment and entry.

This invention also relates to a means and method for quantifying the affect that specific mutations in protease or reverse transcriptase have on replication fitness.

In a further embodiment of the invention , a means and method are provided for quantifying the affect that specific protease and reverse transcriptase mutations have on replication fitness in other viral genes involved in HIV-1 replication, including, but not limited to the gag, pol, and envelope genes.

This invention also relates to the high incidence of patient samples with reduced replication fitness.

This invention relates to the correlation between reduced drug susceptibility and reduced replication fitness.

This invention further relates to the occurrence of viruses with reduced fitness in patients receiving protease inhibitor and/or reverse transcriptase inhibitor treatment.

This invention further relates to the incidence of patient samples with reduced replication fitness in which the reduction in fitness is due to altered protease processing of the gag polyprotein (p55).

This invention further relates to the incidence of protease mutations in patient samples that exhibit low, moderate or normal (wildtype) replication fitness.

This invention further relates to protease mutations that are frequently observed, either alone or in combination, in viruses that exhibit reduced replication capacity.

This invention also relates to the incidence of patient samples with reduced replication fitness in which the reduction in fitness is due to altered reverse transcriptase activity. This invention relates to the occurrence of viruses with reduced replication fitness in patients failing antiretroviral drug treatment. This invention further relates to a means and method for using replication fitness measurements to guide the treatment of HIV-1. This invention further relates to a means and method for using replication fitness measurements to guide the treatment of patients failing antiretroviral drug treatment. This invention further relates to the means and methods for using replication fitness measurements to guide the treatment of patients newly infected with HIV-1.

This invention, provides the means and methods for using replication fitness measurements to guide the treatment of viral diseases, including, but not limited to HIV-1, hepadnaviruses (human hepatitis B virus), flaviviruses (human hepatitis C virus) and herpesviruses (human cytomegalovirus).

In a further embodiment, the invention provides a method for determining replication capacity for a patient's virus comprising:

(a) introducing a resistance test vector comprising a patient derived segment and an indicator gene into a host cell;

(b) culturing the host cell from (a);

(c) harvesting viral particles from step (b) and infecting target host cells;

(d) measuring expression of the indicator gene in the target host cell, wherein the expression of the indicator gene is dependent upon the patient-derived segment;

(e) comparing the expression of the indicator gene from (d) with the expression of the indicator gene measured when steps (a) through (d) are carried out in a control resistance test vector; and

(f) normalizing the expression of the indicator gene by measuring an amount of virus in step (c).

As used herein, "patient-derived segment" encompasses segments derived from human and various animal species. Such species include, but are not limited to chimpanzees, horses, cattles, cats and dogs.

Patient-derived segments can also be incorporated into resistance test vectors using any of several alternative cloning techniques as set forth in detail in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is hereby incorporated by reference. For example, cloning via the introduction of class II restriction sites into both the plasmid backbone and the patient-derived segments or by uracil DNA glycosylase primer cloning.

The patient-derived segment may be obtained by any method of molecular cloning or gene amplification, or modifications thereof, by introducing patient sequence acceptor sites, as described below, at the ends of the patient-derived segment to be introduced into the resistance test vector. For example, in a gene amplification method such as PCR, restriction sites corresponding to the patient-sequence acceptor sites can be incorporated at the ends of the primers used in the PCR reaction. Similarly, in a molecular cloning method such as cDNA cloning, said restriction sites can be incorporated at the ends of the primers used for first or

second strand cDNA synthesis, or in a method such as primer-repair of DNA, whether cloned or uncloned DNA, said restriction sites can be incorporated into the primers used for the repair reaction. The patient sequence acceptor sites and primers are designed to improve the representation of patient-derived segments. Sets of resistance test vectors having designed patient sequence acceptor sites provide representation of patient-derived segments that may be underrepresented in one resistance test vector alone.

"Resistance test vector" means one or more vectors which taken together contain DNA comprising a patient-derived segment and an indicator gene. Resistance test vectors are prepared as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319), which is hereby incorporated by reference, by introducing patient sequence acceptor sites, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into indicator gene viral vectors at the patient sequence acceptor sites. Alternatively, a resistance test vector (also referred to as a resistance test vector system) is prepared by introducing patient sequence acceptor sites into a packaging vector, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into the packaging vector at the patient sequence acceptor sites and co-transfecting this packaging vector with an indicator gene viral vector.

"Indicator or indicator gene," as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) refers to a nucleic acid encoding a protein, DNA or RNA structure that either directly or through a reaction gives rise to a measurable or noticeable aspect, e.g. a color or light of a measurable wavelength or in the case of DNA or RNA used as an indicator a change or generation of a specific DNA or RNA structure. Preferred examples of an indicator gene is the E. coli lacZ gene which encodes beta-galactosidase, the luc gene which encodes luciferase either from, for example, Photonis pyralis (the firefly) or Renilla reniformis (the sea pansy), the E. coli phoA gene which encodes alkaline phosphatase, green fluorescent protein and the bacterial CAT gene which encodes chloramphenicol acetyltransferase. The indicator or indicator gene may be functional or non-functional as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319).

The phenotypic drug susceptibility and resistance tests of this invention may be carried out in one or more host cells as described in US Patent Number 5,837,464 (International Publication Number WO 97/27319) which is incorporated herein by reference. Viral drug susceptibility is determined as the concentration of the anti-viral agent at which a given percentage of indicator gene expression is inhibited (e.g. the IC50 for an anti-viral agent is the concentration at which 50% of

indicator gene expression is inhibited). A standard curve for drug susceptibility of a given anti-viral drug can be developed for a viral segment that is either a standard laboratory viral segment or from a drug-naive patient (i.e. a patient who has not received any anti-viral drug) using the method described in the aforementioned patent. Correspondingly, viral drug resistance is a decrease in viral drug susceptibility for a given patient compared to such a given standard or when making one or more sequential measurements in the same patient over time, as determined by decreased susceptibility in virus from later time points compared to that from earlier time points.

The antiviral drugs being added to the test system are added at selected times depending upon the target of the antiviral drug. For example, in the case of HIV protease inhibitors, including saquinavir, ritonavir, indinavir, nelfinavir and amprenavir, they are added to packaging host cells at the time of or shortly after their transfection with a resistance test vector, at an appropriate range of concentrations. HIV reverse transcriptase inhibitors, including AZT, ddI, ddC, d4T, 3TC, abacavir, nevirapine, delavirdine and efavirenz are added to target host cells at the time of or prior to infection by the resistance test vector viral particles, at an appropriate range of concentration. Alternatively, the antiviral drugs may be present throughout the assay. The test concentration is selected from a range of concentrations which is typically between about 8×10^{-6}

μM and about 2mM and more specifically for each of the following drugs: saquinavir, indinavir, nelfinavir and amprenavir, from about 2.3 X 10^{-5} μM to about 1.5 μM and ritonavir, from about 4.5 X 10^{-5} μM to about 3 μM.

In another embodiment of this invention, a candidate PRI antiretroviral compound is tested in the phenotypic drug susceptibility and resistance test using the resistance test vector comprising PR having a mutation at codon 88 to a serine. The candidate antiviral compound is added to the test system at an appropriate range of concentrations and at the transfection step. Alternatively, more than one candidate antiviral compound may be tested or a candidate antiviral compound may be tested in combination with an approved antiviral drug such as AZT, ddI, ddC, d4T, 3TC, abacavir, delavirdine, nevirapine, efavirenz, saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, or a compound which is undergoing clinical trials such as adefovir and ABT-378. The effectiveness of the candidate antiviral will be evaluated by measuring the expression or inhibition of the indicator gene. In another aspect of this embodiment, the drug susceptibility and resistance test may be used to screen for viral mutants. Following the identification of mutants resistant to either known antiretrovirals or candidate antiretrovirals the resistant mutants are isolated and the DNA is analyzed. A library of viral resistant mutants can thus be assembled enabling the screening of candidate PRI antiretrovirals, alone or in combination. This will enable one of ordinary skill to

identify effective PRI antiretrovirals and design effective therapeutic regimens.

The structure, life cycle and genetic elements of the viruses which could be tested in the drug susceptibility and resistance test of this invention would be known to one of ordinary skill in the art. It is useful to the practice of this invention, for example, to understand the life cycle of a retrovirus, as well as the viral genes required for retrovirus rescue and infectivity. Retrovirally infected cells shed a membrane virus containing a diploid RNA genome. The virus, studded with an envelope glycoprotein (which serves to determine the host range of infectivity), attaches to a cellular receptor in the plasma membrane of the cell to be infected. After receptor binding, the virus is internalized and uncoated as it passes through the cytoplasm of the host cell. Either on its way to the nucleus or in the nucleus, the reverse transcriptase molecules resident in the viral core drive the synthesis of the double-stranded DNA provirus, a synthesis that is primed by the binding of a tRNA molecule to the genomic viral RNA. The double-stranded DNA provirus is subsequently integrated in the genome of the host cell, where it can serve as a transcriptional template for both mRNAs encoding viral proteins and virion genomic RNA, which will be packaged into viral core particles. On their way out of the infected cell, core particles move through the cytoplasm, attach to the inside of the plasma membrane of the newly infected cell, and bud, taking with them tracts of membrane containing the virally encoded

envelope glycoprotein gene product. This cycle of infection - reverse transcription, transcription, translation, virion assembly, and budding - repeats itself over and over again as infection spreads.

The viral RNA and, as a result, the proviral DNA encode several cis-acting elements that are vital to the successful completion of the viral lifecycle. The virion RNA carries the viral promoter at its 3' end. Replicative acrobatics place the viral promoter at the 5' end of the proviral genome as the genome is reverse transcribed. Just 3' to the 5' retroviral LTR lies the viral packaging site. The retroviral lifecycle requires the presence of virally encoded transacting factors. The viral-RNA-dependent DNA polymerase (*pol*)-reverse transcriptase is also contained within the viral core and is vital to the viral life cycle in that it is responsible for the conversion of the genomic RNA to the integrative intermediate proviral DNA. The viral envelope glycoprotein, *env*, is required for viral attachment to the uninfected cell and for viral spread. There are also transcriptional *trans*-activating factors, so called transactivators, that can serve to modulate the level of transcription of the integrated parental provirus. Typically, replication-competent (non-defective) viruses are self-contained in that they encode all of these trans-acting factors. Their defective counterparts are not self-contained.

In the case of a DNA virus, such as a hepadnavirus, understanding the life cycle and viral genes required for infection is useful to the practice of this invention. The process of HBV entry has not been well defined. Replication of HBV uses an RNA intermediate template. In the infected cell the first step in replication is the conversion of the asymmetric relaxed circle DNA (rc-DNA) to covalently closed circle DNA (cccDNA). This process, which occurs within the nucleus of infected liver cells, involves completion of the DNA positive-strand synthesis and ligation of the DNA ends. In the second step, the cccDNA is transcribed by the host RNA polymerase to generate a 3.5 kB RNA template (the pregenome). This pregenome is complexed with protein in the viral core. The third step involves the synthesis of the first negative-sense DNA strand by copying the pregenomic RNA using the virally encoded P protein reverse transcriptase. The P protein also serves as the minus strand DNA primer. Finally, the synthesis of the second positive-sense DNA strand occurs by copying the first DNA strand, using the P protein DNA polymerase activity and an oligomer of viral RNA as primer. The pregenome also transcribes mRNA for the major structural core proteins.

The following flow chart illustrates certain of the various vectors and host cells which may be used in this invention. It is not intended to be all inclusive.

Vectors

Indicator gene cassette (functional/nonfunctional indicator gene) + Viral vector (genomic or subgenomic)

↓

Indicator Gene Viral Vector
(functional/nonfunctional indicator gene)

↓ + Patient sequence acceptor sites
+ Patient-derived segments

Resistance Test Vector
(patient-derived segments + indicator gene)

Host Cells

Packaging Host Cell - transfected with packaging

expression vectors

Resistance Test Vector Host Cell - a packaging host cell transfected with a resistance test vector

Target Host Cell - a host cell to be infected by a resistance test vector viral particle produced by the resistance test vector host cell

Resistance Test Vector

"Resistance test vector" means one or more vectors which taken together contain DNA or RNA comprising a patient-derived segment and an indicator gene. In the case where the resistance test vector comprises more than one vector the patient-derived segment may be contained in one vector and the indicator gene in a different vector. Such a resistance test vector comprising more than one vector is referred to herein as a resistance test vector system for purposes of clarity but is nevertheless understood to be a resistance test vector. The DNA or RNA of a resistance test vector may thus be contained in one or more DNA or RNA molecules. In one embodiment, the resistance test vector is made by insertion of a patient-derived segment into an indicator gene viral vector. In another embodiment, the resistance test vector is made by insertion of a patient-derived segment into a packaging vector while the indicator gene is contained in

a second vector, for example an indicator gene viral vector. As used herein, "patient-derived segment" refers to one or more viral segments obtained directly from a patient using various means, for example, molecular cloning or polymerase chain reaction (PCR) amplification of a population of patient-derived segments using viral DNA or complementary DNA (cDNA) prepared from viral RNA, present in the cells (e.g. peripheral blood mononuclear cells, PBMC), serum or other bodily fluids of infected patients. When a viral segment is "obtained directly" from a patient it is obtained without passage of the virus through culture, or if the virus is cultured, then by a minimum number of passages to essentially eliminate the selection of mutations in culture. The term "viral segment" refers to any functional viral sequence or viral gene encoding a gene product (e.g., a protein) that is the target of an anti-viral drug. The term "functional viral sequence" as used herein refers to any nucleic acid sequence (DNA or RNA) with functional activity such as enhancers, promoters, polyadenylation sites, sites of action of trans-acting factors, such as *tar* and RRE, packaging sequences, integration sequences, or splicing sequences. If a drug were to target more than one functional viral sequence or viral gene product then patient-derived segments corresponding to each said viral gene would be inserted in the resistance test vector. In the case of combination therapy where two or more anti-virals targeting two different functional viral sequences or viral gene products are being evaluated,

patient-derived segments corresponding to each functional viral sequence or viral gene product would be inserted in the resistance test vector. The patient-derived segments are inserted into unique restriction sites or specified locations, called patient sequence acceptor sites, in the indicator gene viral vector or for example, a packaging vector depending on the particular construction being used as described herein.

As used herein, "patient-derived segment" encompasses segments derived from human and various animal species. Such species include, but are not limited to chimpanzees, horses, cattles, cats and dogs.

Patient-derived segments can also be incorporated into resistance test vectors using any of several alternative cloning techniques. For example, cloning via the introduction of class II restriction sites into both the plasmid backbone and the patient-derived segments or by uracil DNA glycosylase primer cloning (refs).

The patient-derived segment may be obtained by any method of molecular cloning or gene amplification, or modifications thereof, by introducing patient sequence acceptor sites, as described below, at the ends of the patient-derived segment to be introduced into the resistance test vector. For example, in a gene amplification method such as PCR, restriction sites corresponding to the patient-sequence acceptor sites can

be incorporated at the ends of the primers used in the PCR reaction. Similarly, in a molecular cloning method such as cDNA cloning, said restriction sites can be incorporated at the ends of the primers used for first or second strand cDNA synthesis, or in a method such as primer-repair of DNA, whether cloned or uncloned DNA, said restriction sites can be incorporated into the primers used for the repair reaction. The patient sequence acceptor sites and primers are designed to improve the representation of patient-derived segments. Sets of resistance test vectors having designed patient sequence acceptor sites provide representation of patient-derived segments that would be underrepresented in one resistance test vector alone.

Resistance test vectors are prepared by modifying an indicator gene viral vector (described below) by introducing patient sequence acceptor sites, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into indicator gene viral vectors at the patient sequence acceptor sites. The resistance test vectors are constructed from indicator gene viral vectors which are in turn derived from genomic viral vectors or subgenomic viral vectors and an indicator gene cassette, each of which is described below. Resistance test vectors are then introduced into a host cell. Alternatively, a resistance test vector (also referred to as a resistance test vector system) is prepared by introducing patient sequence acceptor sites

into a packaging vector, amplifying or cloning patient-derived segments and inserting the amplified or cloned sequences precisely into the packaging vector at the patient sequence acceptor sites and co-transfecting this packaging vector with an indicator gene viral vector.

In one preferred embodiment, the resistance test vector may be introduced into packaging host cells together with packaging expression vectors, as defined below, to produce resistance test vector viral particles that are used in drug resistance and susceptibility tests that are referred to herein as a "particle-based test." In an alternative preferred embodiment, the resistance test vector may be introduced into a host cell in the absence of packaging expression vectors to carry out a drug resistance and susceptibility test that is referred to herein as a "non-particle-based test." As used herein a "packaging expression vector" provides the factors, such as packaging proteins (e.g. structural proteins such as core and envelope polypeptides), transacting factors, or genes required by replication-defective retrovirus or hepadnavirus. In such a situation, a replication-competent viral genome is enfeebled in a manner such that it cannot replicate on its own. This means that, although the packaging expression vector can produce the trans-acting or missing genes required to rescue a defective viral genome present in a cell containing the enfeebled genome, the enfeebled genome cannot rescue itself.